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(54) Utilization of enzymes.

(57) One or more vehicles, which may be acceptable to enter the mouth, contain at least one of two cooperating enzymes plus means to link them together. One enzyme generates an intermediate which is used by the other enzyme to generate a substance preferably hypohalite, which acts at the target site. The means to link then causes the intermediate to be formed peroxidase to the enzyme which uses it.

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Horseradish peroxidase is commercially available and could be used in conjunction with either of these oxidases. A further possibility is lactoperoxidase.

Various applications of the invention are envisaged, although the invention is not limited to these. One application which is particularly envisaged is to attack species of the supragingival oral microflora. For this and other applications the vehicle(s) preferably contain means for attaching the enzymes to a target site.

The oral microflora is a complex ecosystem which contains a wide variety of microbial species. One of these species may be selected as the target site. However, the effect of targeting to one species will be to attack both that species and other species which occur in close proximity to it. Thus, by delivering to one species which occurs in dental plaque, cytotoxic agents will be delivered to the plaque and will act against all the species which occur together in the plaque, including those responsible for plaque formation. Extracellular dextran produced by such organisms could itself be used as a target site.

One possible target site is Streptococcus mutans. This has been identified as an important contributor to dental plaque, and has been shown to be capable of inducing clinical caries lesions in germ free animals when established as a mono-infection. S. mutans has the ability to utilise dietary carbohydrate for the synthesis of an insoluble polysaccharide matrix, facilitating attachment to, and colonisation of, hard surfaces, as well as production of acids capable of the dissolution of enamel. These characteristics have been identified as important virulence determinants. Although other species and genera have also proved capable of both acid and plaque production, or even of caries initiation in the germ free animal, S. mutans is widely recognised as at least one significant cause of tooth decay because of the scale of its acid and polysaccharide production.

Other species which may be selected as the target species are S. sanguis, A. viscosus and A. naeslundii. These are all present in dental plaque as a substantial proportion of the species normally found in dental plaque. Because of frequent occurrence, these three may be preferred as target site.

Another application is to attack species of the subgingival microflora responsible for periodontal disease. The target species could well be Bacteroides gingivalis.

A possible cosmetic application is the reduction of stain on teeth. In this application enzymes are used which produce a material with a bleaching function, such as hypohalite ion. The target site is at the tooth surface where staining may be present.

For any oral application (dental care) it would be necessary for the vehicle(s) in the product to be acceptable to enter the mouth, e.g. vehicles suitable for topical application in the mouth.

Another application is to attack human tumour cells, notably in bone marrow which has been removed temporarily from the body of a patent undergoing radiotherapy.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention may be implemented in various ways. Some of these are illustrated in the accompanying drawings which are all schematic diagrams of linking arrangements. G.Ox denotes glucose oxidase. HRP denotes horseradish peroxidase. PEI denotes polyethyleneimine.

In the drawings:

Fig. 1 shows two enzymes linked by means of polyethyleneimine;

Figs. 2 and 3 each show two enzymes linked together by means of antibodies;

Figs. 4 to 8 each show one of the above complexes attached to a target;

Fig. 9 shows two enzymes linked together and attached to a target by means of antibody fragments;

Figs. 10 and 11 each show enzymes linked through a target;

Fig. 12 shows enzymes linked by a target specific antibody.

DETAILED DESCRIPTION OF EMBODIMENTS

This invention requires two enzymes to be linked together. The enzymes which are linked may be attached to a target. Attachment is preferably accomplished by means of an antibody or antibody fragment which binds to the target site.

In certain significant forms of this invention, the linking means extends between the enzymes and couples the enzymes together otherwise than through the target site (which may be a cell) or through a single whole antibody which binds directly to the target site. This makes it possible to avoid subjecting a target-specific antibody to artificial chemical reactions used to effect conjugation of enzymes to the antibody through covalent bond formation. Secondly, linking the enzymes otherwise than through the target site or a target-specific antibody can make it easier to control the distribution of enzymes and get the two kinds of enzyme in proximity to each other, so that the intermediate which is the product of one enzyme is generated in proximity to the other enzyme.

One possibility is that the linking means is a carrier material to which both enzymes are conjugated chemi-

ferred arrangement utilises an antibody fragment to bind to a target site and further fragments to bind enzymes to the first fragment.

The first antibody fragment which binds to a target site may be an Fv fragment of an antibody to the desired target. Such a fragment contains only the variable domains of light and heavy chains of an antibody. The fragment could possibly be an $F(ab)_2$ fragment which would provide two combining sites. It might alternatively be as little as a single variable domain of one chain of an antibody.

Techniques for efficient production of biologically active antibody fragments in E. coli were described by A Skevia and A. Pluckthun (1988), Science, 240, 1038; M. Better et al, (1988), Science, 240, 1041; and E.S. Ward et al, (1989), Nature, 341, 544. The cloning and expression of genes encoding antibody fragments in E. coli is also described in published European application EP-A-368684 (Medical Research Council).

We prefer that a first antibody fragment has an additional peptide chain appended to it, and further fragments bind the enzymes to this peptide chain. Bacteria can be made to produce a variable domain with an extra peptide chain already attached to the C-terminus of the domain. This can be achieved by standard genetic techniques. For example, a gene encoding a variable domain can easily be lengthened by means of site directed mutagenesis techniques, using in vitro synthesised oligonucleotides which encode the peptide to be appended to the variable region. Site directed mutagenesis is now a widely used technique, and adequate protocols can be found in several published books, for example in Sambrook et al, (1989), Molecular Cloning, 2nd edition, Cold Spring Harbour Laboratory Press, New York.

An attached extra peptide provides a very convenient "handle" for the attachment of the therapeutic agent.

Further antibody fragments which bind enzymes to the first fragment are preferably a second antibody fragment able to bind to an enzyme by antibody-antigen binding, and an $F(ab)_2$ fragment (which is bivalent) able to bind to the first and second antibody fragments, especially to antigenic peptides appended to the first and second antibody fragments.

Fig. 9 illustrates a preferred arrangement in which antibody fragments are utilised.

For attaching to the target 40 which has antigenic sites 42 there is an Fv antibody fragment 44 with several repeats of a peptide 46 appended to the distal (c-terminal) end of one of the two chains in the Fv fragment 44.

Glucose oxidase (G.Ox) and horseradish peroxidase (HRP) are each bound by a respective Fv fragment 48,50 with specificity for the enzyme concerned, and with a single repeat of the same peptide 46 appended to one chain of the Fv fragment.

The peptides 46 appended to the anti-enzyme Fv fragments 48,50 become linked to peptides 46 on the anti-target Fv fragment by $F(ab)_2$ fragments 52 which bind specifically to these peptides. Since the $F(ab)_2$ fragments are divalent they can form a bridge attaching an anti-enzyme fragment, with attached enzyme, to the anti-target fragment 44.

Within this invention it is possible that the enzymes are bound to a target site and so become linked but are not linked more directly. Arrangements of this kind are shown in Figs. 10 and 11.

In Fig. 10 numeral 20 denotes a target which is S. mutans with antigenic sites 22. Numeral 54 denotes anti-S. mutans antibody which has glucose oxidase (G.Ox) conjugated to it. Numeral 56 denotes anti-S. mutans antibody which has horseradish peroxidase (HRP) conjugated to it.

In Fig. 11 the need for chemical conjugation is avoided by the use of multiple antibodies.

Numeral 26 denotes rabbit anti-S. mutans antibody. Numeral 16 denotes rabbit anti-glucose oxidase, bound to that enzyme. Numeral 58 denotes rabbit anti-horseradish peroxidase bound to horseradish peroxidase. Numeral 28 denotes goat anti-rabbit immunoglobulin which acts to bind antibodies 16 and 58 to anti-target antibodies 26.

Fig. 12 shows another arrangement in which both enzymes are chemically conjugated to a single antibody, which also binds to the target.

Antibodies used in this invention may be polyclonal or monoclonal. Where an antibody of one specificity and an antibody of a different specificity are used together, it is possible that one antibody would be monoclonal while the other antibody was polyclonal.

If a plurality of polyclonal antibodies are used, it may be found desirable to distribute the enzymes and antibodies between more than one vehicle in the product , so that the full complexes of both enzymes and antibodies do not form until the time of use. We have found that during storage, large complexes with polyclonal antibodies are prone to suffer a reduction in their ability to bind to a target site.

Distribution of constituents of the complex between a plurality of vehicles may be unnecessary if monoclonal antibodies are employed. In general, monoclonal antibodies would form smaller complexes and during storage would be expected to retain their activity better than complexes formed with polyclonal antibodies. This is also true when antibody fragments are used. An advantage of antibody fragments is that the complexes which form by antigen - antibody binding are fairly small and more stable than complexes with whole antibodies.

When a product has the enzymes and one or more antibodies distributed between two vehicles, one of

5. 1ml of filter sterilised glucose oxidase/sheep anti-rabbit conjugate, at 1/10 dilution in PBS at pH 8 was now added, giving a final dilution of 1/100 after addition. Again, the suspension was incubated at room temperature for 20 minutes, then the cells were centrifuged into a pellet and washed 3 times with PBS.
6. The cells were resuspended in 10ml of filter sterilised PBS at pH 6.5 containing 15µg/ml potassium iodide and 5% w/v D-glucose.
7. The resulting suspension was incubated at 37°C for 24 hours. 0.5ml samples were taken immediately on mixing, and at intervals. The bacteria in each sample were separated by centrifugation into a pellet which was washed three times with PBS. The viable cells in each sample were assayed by the method of Miles, Misra and Irwin J. Hygiene 38, 732-749, (1938). The combinations of materials added, and the counts of viable cells are set out in the following Table.
8. Samples of the suspension produced in step 6 were assayed colorimetrically for the simultaneous presence of both enzymes bound to the cells. The cells were centrifuged at 4000rpm for 5 minutes and then washed 3 times by resuspending the pellet in 3ml of PBS, and centrifuging again. After the last centrifugation the cells were resuspended in 0.5ml of PBS containing 100mM glucose and 1µg/ml of tetramethyl benzidine. This system develops colour only where glucose oxidase and peroxidase are present together. Colour was allowed to develop for 5 minutes, then stopped by addition of 50µl of 0.2M HCl. Optical densities were determined and are included in Table 1.

As can be seen from the Table, the cells generally survive in the presence of antibodies to them (experiment A). When glucose oxidase conjugate is present, but unable to bind to the S. mutans cells (experiment G) it displays a cell killing effect. It displays a greater cell killing effect if bound to the target S. mutans cells (experiment D). Surprisingly, horseradish peroxidase conjugate produced some cell killing without glucose oxidase (experiment F).

In experiment C both conjugates were present and able to complex together.

Experiment C shows both conjugates present and able to complex together. Thus this experiment had both enzymes present and linked together. There was cell killing and it was greater than when glucose oxidase conjugate was present, but unbound, (experiment G) or when horseradish peroxidase alone was bound to the target (experiment F).

Even greater cell killing is achieved by experiment B which contains all the components to enable both conjugates to attach together and also attach to the target cells. The number of surviving cells dropped dramatically within two hours, and eventually fell to zero.

Example 2

The procedure of Example 1 was repeated, using two different dilutions of the glucose oxidase/sheep anti-rabbit conjugate. In some experiments the conjugate used was 1/10 dilution in PBS at pH 8, so that after addition the final dilution of the conjugate was 1/100. In other experiments the conjugate was used at 1/160 dilution, so that after addition the final dilution was 1/1600.

The combinations of materials added, the counts of viable cells, and optical densities from the colorimetric assay are set out in the following Table.

It can be seen from Table 2 that cells survive in the presence of antibodies to them (experiment A) and that the dilute glucose oxidase conjugate displays little cell killing activity in the absence of peroxidase (experiments F and G).

Experiments C and E both used linked enzymes, because there was a complex of peroxidase conjugate with glucose oxidase conjugate. In both experiments there was cell killing, although experiment E with dilute glucoseoxidase conjugate gave markedly less cell killing than experiment C using the more concentrated glucose oxidase conjugate. Much faster cell killing was observed with complex bound to the S. mutans cells (experiments B and D). The effectiveness of the bound complex with the dilute glucose oxidase conjugate (experiment D) was particularly notable.

Example 3

This example demonstrates the covalent conjugation of glucose oxidase (G.Ox) and horseradish peroxidase (HRP) to polyethyleneimine (PEI) of molecular weight 50,000-60,000 (ex Aldrich). Covalent coupling is achieved by periodate oxidation of pendant glycosyl chains on the enzymes, to generate aldehyde groups which react with amino groups on the PEI. These chemical links (Schiff's bases) are subsequently stabilised by reduction with sodium borohydride, which simultaneously removes unused reactive aldehydes (by reduction to alcohols) to prevent further chemical coupling.

Part 1 : Preparation of the dual enzyme-PEI (DEPEI) conjugate

1. Oxidation of the enzymes

G.Ox (10mg) was dissolved in 2.0ml distilled water, and then mixed with 0.2ml of a freshly prepared 0.1M solution of sodium metaperiodate in distilled water. The mixture was stirred for 20 minutes in the dark at ambient temperature, after which it was dialysed overnight at 4°C against 1mM sodium acetate/acetic acid buffer, pH 4.4 (1 litre).

HRP was oxidised in the same way, except that it was at a concentration of 2.5mg in 2ml distilled water.

2. Conjugation of oxidised enzymes to PEI

The pH of each oxidised enzyme solution was raised by the addition of 0.05ml of 0.2M sodium carbonate/bicarbonate buffer, pH 9.5. At this point, 0.2ml of the oxidised G.Ox solution was mixed with 0.2ml of a 100µg/ml solution of PEI in 0.01M sodium carbonate/bicarbonate buffer at pH 9.5. The mixture was kept at ambient temperature in the dark for 30 minutes. 0.2ml of the oxidised HRP was then added, and the reaction was continued for a further 2 hours (still in the dark). At the end of this stage the reaction was stopped by the addition of 0.03ml of a 5mg/ml solution of sodium borohydride in distilled water. Part 2 : It was demonstrated in vitro that the DEPEI conjugate would attach to S. mutans cells

A culture of S. mutans cells (as described in the earlier examples) was washed by centrifugation and resuspension (4 times) in PBS and finally resuspended in the original volume of PBS. Samples of this bacterial suspension (0.2ml) were mixed with equal volumes of PBS containing 1% bovine serum albumin and 0.15% Tween 20 (PBST/BSA), held at ambient temperature for 30 minutes, sedimented again by centrifugation and then resuspended in a solution of DEPEI conjugate diluted 1/50 in PBST/BSA (at pH 8.0).

The S. mutans cells and DEPEI conjugate were left in contact for 30 minutes at ambient temperature, after which the cells were sedimented and resuspended in PBST/BSA 3 times. An identical control sample was subjected to the same procedure, but without the addition of any DEPEI conjugate.

To demonstrate the presence of bound DEPEI on the surface of the cells, the sedimented pellet was resuspended in 0.5ml of a solution of tetramethyl benzidine (TMB, Sigma) and glucose in 0.1M phosphate/citrate buffer, pH 6.5 (TMB at 100µg/ml and glucose at 27mg/ml). This mixture was maintained at ambient temperature for 5 minutes, after which the cells were centrifuged to a pellet and a 0.2ml sample of the supernatant fluid was transferred to a microtitre plate. 0.05ml of 2M hydrochloric acid was added, then the optical density of the fluid was measured. Optical densities were:

Experimental sample : 1.07

Control (no DEPEI) : 0.01

This experiment was repeated with varying pH for the solution of DEPEI conjugate. It was found that if a pH of 5.5 to 7.5 was used the optical density rose slightly, indicating more binding. If pH was 8.5 or greater the optical density fell sharply. Changing the ionic strength by addition of sodium chloride also affected binding, indicating that the binding of DEPEI to S. mutans cells is by ionic interactions.

to 14, to prepare a product for topical application.

Fig. 4.

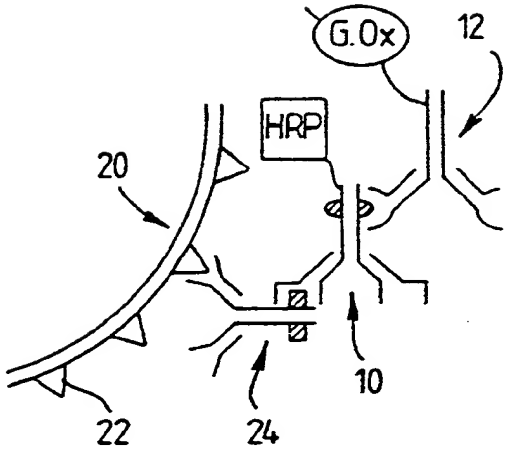


Fig. 5.

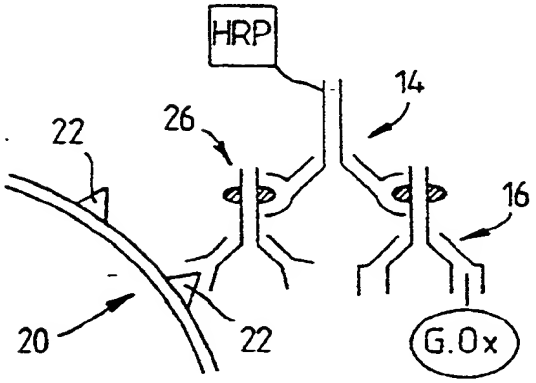


Fig. 9.

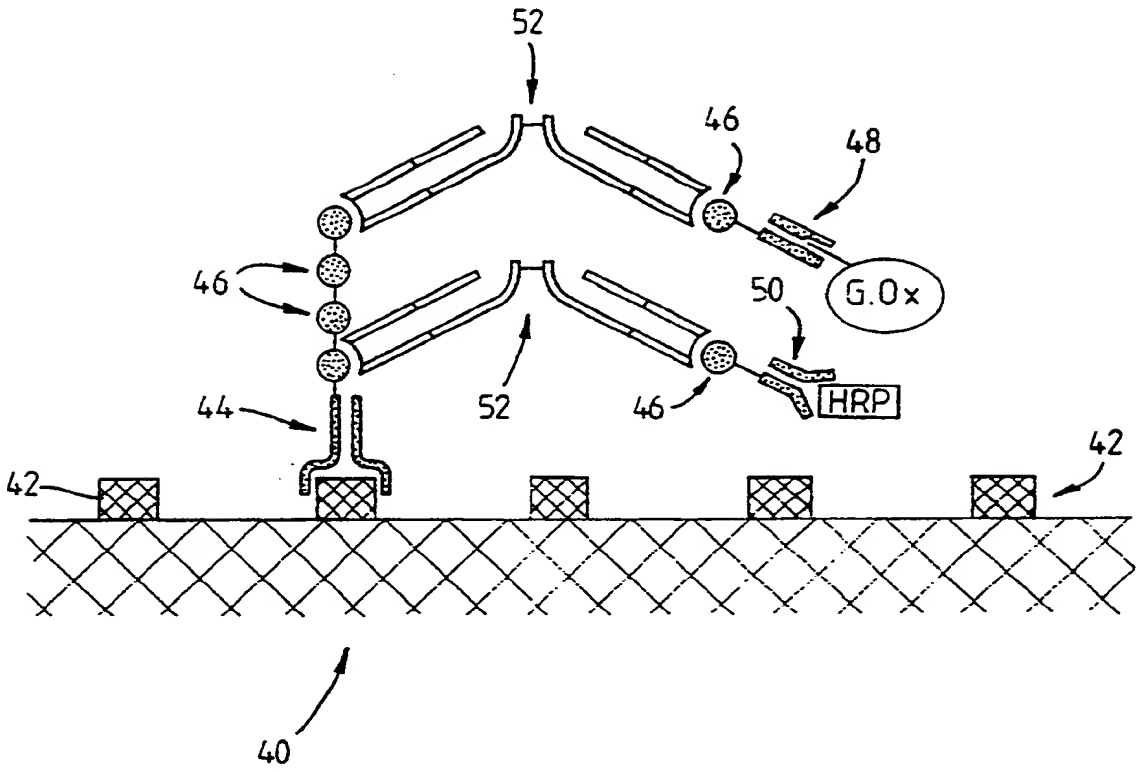
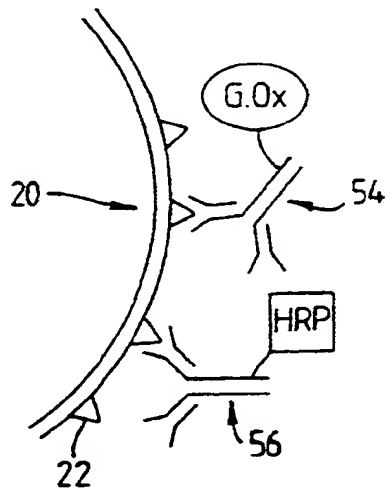


Fig. 10.



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EUROPEAN SEARCH REPORT

Application Number

EP 91 30 2396

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X,P Y	WO-A-9 003 185 (IDEON CORP.) * Page 5, lines 28-34; page 9, lines 5-35; page 11, line 34 - page 12, line 4; claim 5 *	1-18	A 61 K 47/48 A 61 K 7/28
Y	US-A-4 578 265 (M.A. PELLICO) * Column 6, lines 5-36; claims *	1-18	
P,Y	FR-A-2 651 433 (D. DANA) * Page 1, lines 1-17; page 2, lines 3-9,18-20; claims *	1-18	
P,X	WO-A-9 100 112 (BRUNSWICK CORP.) * Page 2, line 31 - page 3, line 19; page 6, lines 8-25; claims *	1-9,11-13,18	
A	WO-A-8 801 178 (THE DOW CHEM. CO.) * Pages 89-91; claims 1-7 *	1-18	
A	STN FILE SERVER & FILE MEDLINE, AN=81192354; R.B. JOHNSON, Jr. et al.: "Comparison of glucose oxidase and peroxidase as labels for antibody in enzyme-linked immunosorbent assay", & J. IMMUNOASSAY, (1980), 1(1), 27-37 * Whole abstract *	1-18	TECHNICAL FIELDS SEARCHED (Int. Cl.5) A 61 K
X,Y	SNT FILE SERVER & FILE CA, CHEMICAL ABSTRACTS, vol. 105, no. 17, abstract no. 145834w, Columbus, Ohio, US; J. PENE et al.: "In vitro cytolysis of myeloma tumor cells with glucose oxidase and lactoperoxidase antibody conjugates", & BIOCHEM. INT., 13(2), 233-43 * Whole abstract *	1-18	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	23-07-1991	BERTE M.J.

EPO FORM 1503 03.82 (P0401)

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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